EMBO reports

scientific report

Endosomal hyperacidification in cystic fibrosis is due to defective nitric oxide–cylic GMP signalling cascade

Jens F. Poschet[1], Joseph A. Fazio[1]*, Graham S. Timmins[4], Wojciech Ornatowski[1], Elizabeth Perkett[2], Monica Delgado[1] & Vojo Deretic[1,3]+*

Departments of [1]Molecular Genetics & Microbiology, [2]Pediatry, and [3]Cell Biology & Physiology, School of Medicine, and [4]College of Pharmacy, Health Sciences Center, University of New Mexico, Albuquerque, New Mexico, USA

Endosomal hyperacidification in cystic fibrosis (CF) respiratory epithelial cells is secondary to a loss of sodium transport control owing to a defective form of the CF transmembrane conductance regulator CFTR. Here, we show that endosomal hyperacidification can be corrected by activating the signalling cascade controlling sodium channels through cyclic GMP. Nitric oxide (NO) donors corrected the endosomal hyperacidification in CF cells. Stimulation of CF cells with guanylate cyclase agonists corrected the pH in endosomes. Exposure of CF cells to an inhibitor of cGMP-specific phosphodiesterase PDE5, Sildenafil, normalized the endosomal pH. Treatment with Sildenafil reduced secretion by CF cells of the proinflammatory chemokine interleukin 8 following stimulation with *Pseudomonas aeruginosa* products. Thus, the endosomal hyperacidification and excessive proinflammatory response in CF are in part due to deficiencies in NO- and cGMP-regulated processes and can be pharmacologically reversed using PDE5 inhibitors.

Keywords: cystic fibrosis; endosome; nitric oxide; cGMP; phosphodiesterase; Sildenafil

EMBO reports (2006) **7**, 553–559. doi:10.1038/sj.embor.7400674

INTRODUCTION

Cystic fibrosis (CF) is a life-shortening inheritable disorder (Rowe *et al*, 2005), caused by mutations in the gene encoding a chloride channel termed the CF transmembrane conductance regulator (CFTR). CF is a syndrome affecting respiratory, gastrointestinal, hepatobiliary and male reproductive tracts (Rowe *et al*, 2005). Patients with identical CFTR mutations often show different clinical presentation, suggesting a role for modifier genes other than CFTR (Cutting, 2005). In keeping with this idea, CFTR exerts pleiotropic effects on transport of other ions, including the amiloride-sensitive epithelial sodium channel (ENaC; Stutts *et al*, 1995), and affects a variety of transport and physiological properties of respiratory cells and tract (Poschet *et al*, 2002a). A link between the CFTR defect and lung pathology, that is, *Pseudomonas aeruginosa* infections, excessive inflammation and irreversible lung damage, remains to be established. It is known however that the proinflammatory chemokine interleukin 8 (IL-8; CXCR-8) is abnormally elevated in CF and is responsible for neutrophil infiltration and subsequent inflammatory cascade (Bonfield *et al*, 1995). Nevertheless, the basis for abnormally high IL-8 and its pathology-inducing instead of infection-resolving function are not known.

In CF, about 70% of the CFTR mutations are ΔF508, which result in improper folding of the CFTR and its aberrant transport, including retention in the endoplasmic reticulum (Riordan, 2005) and increased endocytic removal from the plasma membrane, as well as increased degradation (Gentzsch *et al*, 2004). The CFTR defect not only leads to abnormal chloride transport, but also affects the function of the ENaC, resulting in its increased probability of being open and higher than normal Na^+ currents (Stutts *et al*, 1995). Another important effect of the upregulation of Na^+ transport in CF is the lower than normal pH in a subset of intracellular organelles in CF respiratory epithelial cells (Poschet *et al*, 2001, 2002a,b). This includes the trans-Golgi network (TGN; Chandy *et al*, 2001; Poschet *et al*, 2001, 2002a) and cellubrevin-positive compartments in the endosomal network (Poschet *et al*, 2002a,b) in CF bronchial and tracheal epithelial cells. The aberrant Na^+ transport in CF affects lumenal hyperacidification of TGN and endosomes in CF cells in the following manner (Poschet *et al*, 2001, 2002a,b): (i) the vacuolar H^+ ATPase is sensitive to transmembrane potential build-up, which increases as the protons are pumped into the organellar lumen and positive charges accumulate; (ii) an efflux of Na^+ from the same lumen helps dissipate the membrane potential and release the H^+ ATPase pump from inhibition; and (iii) the cellubrevin-containing endosomes and TGN become overly acidified owing to increased Na^+ channel activity in CFTR-mutant cells.

Departments of [1]Molecular Genetics & Microbiology, [2]Pediatry, and [3]Cell Biology & Physiology, School of Medicine, and [4]College of Pharmacy, Health Sciences Center, University of New Mexico, Albuquerque, New Mexico, 87131, USA
*These authors contributed equally to this work
+Corresponding author. Tel: +1 505 272 0291; Fax: +1 505 272 5309;
E-mail: vderetic@salud.unm.edu

Received 28 September 2005; revised 23 January 2006; accepted 2 March 2006; published online 13 April 2006

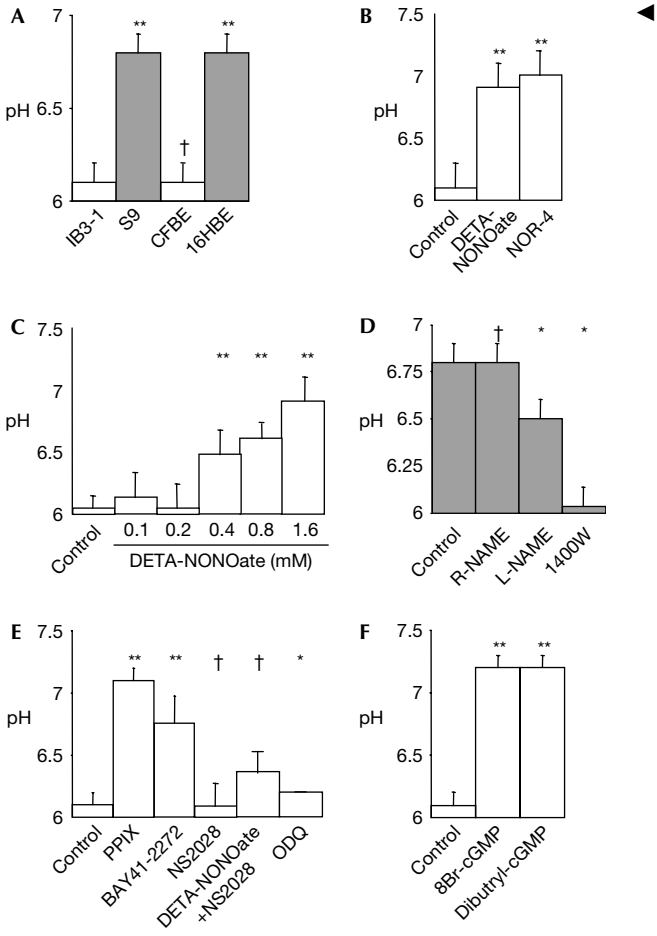

Fig 1 | Nitric oxide corrects hyperacidification in cellubrevin endosomes of cystic fibrosis respiratory epithelial cells. Guanylate cyclase or addition of exogenous cyclic GMP normalizes pH in cellubrevin endosomes of CF cells. (**A**) Lung epithelial cells transfected with cellubrevin GFP pHlourin: IB3-1 (CFBE) pH 6.1 ± 0.1, $n = 18$; S9 (CFTR-corrected IB3-1 cells), pH 6.8 ± 0.1, $n = 18$, $P = 0.0003$; CFBE, pH 6.1 ± 0.1, $n = 28$; 16HBE, pH 6.8 ± 0.1, $n = 28$, $P = 0.0001$. (**B**) CF cells treated with NO• donors and scavengers: IB3-1, pH 6.1 ± 0.1, $n = 18$; IB3-1 + 1 mM DETA-NONOate, pH 6.9 ± 0.1, $n = 10$, $P = 0.0001$; IB3-1 + 1 mM NOR4, pH 7.0 ± 0.2, $n = 10$, $P = 0.0001$. (**C**) Dose–response to the NO• donor DETA-NONOate: IB3-1 + 0.1 mM DETA-NONOate, pH 6.0 ± 0.1, $P = 0.5341$; IB3-1 + 0.2 mM DETA-NONOate, pH 6.1 ± 0.2, $P = 0.9937$; IB3-1 + 0.4 mM DETA-NONOate, pH 6.5 ± 0.2, $P = 0.0018$; IB3-1 + 0.8 mM DETA-NONOate, pH 6.6 ± 0.1, $P = 0.0004$; IB3-1 + 1.6 mM DETA-NONOate, pH 6.9 ± 0.2, $P = 0.0001$. In all experiments, $n = 5$. (**D**) Respiratory cells treated with nitric oxide synthase inhibitors: S9, pH 6.8 ± 0.1, $n = 18$; S9 + 1 mM R-NAME, pH 6.8 ± 0.1, $n = 11$, $P = 0.9333$; S9 + 1 mM L-NAME, pH 6.5 ± 0.1, $n = 10$, $P = 0.0492$; S9 + 10 μM 1400W, pH 6.0 ± 0.2, $n = 5$, $P = 0.0161$. (**E**) CF cells treated with guanylate cyclase agonists (PPIX and BAY41-2272) and antagonists (NS2028 and ODQ): IB3-1 control, pH 6.1 ± 0.1, $n = 18$; IB3-1 + 10 μM PPIX, pH 7.1 ± 0.1, $n = 10$, $P = 0.0001$; IB3-1 + 10 μM BAY41-2272, pH 6.7 ± 0.2, $n = 5$, $P = 0.0004$; IB3-1 + 10 μM NS2028, pH 6.1 ± 0.1, $n = 10$, $P = 0.9385$; IB3-1 + 1 mM DETA-NONOate + 10 μM NS2028, pH 6.4 ± 0.2, $n = 10$, $P = 0.2639$; IB3-1 + 10 μM ODQ, pH 6.2 ± 0.0, $n = 5$, $P = 0.0161$. (**F**) CF cells treated with exogenous cGMP: IB3-1, pH 6.1 ± 0.1, $n = 18$; IB3-1 + 100 μM 8Br-cGMP, pH 7.2 ± 0.1, $n = 10$, $P = 0.0001$; IB3-1 + 100 μM dibutyryl cGMP, pH 7.2 ± 0.1, $n = 10$, $P = 0.0001$. Open bars, CF cells; grey bars, CFTR-corrected or normal cells. CF, cystic fibrosis; CFBE, CF bronchial epithelial cells; CFTR, CF transmembrane conductane regulator; GFP, green fluorescent protein; HBE, normal human bronchial epithelial cells; NO, nitric oxide; PPIX, protoporphyrin IX.

Although the evidence points to a central role of ENaC dysregulation in CF, it is not understood how CFTR affects ENaC activity. It has been reported that one of the consequences of CFTR deficiency is a reduced level of inducible nitric oxide synthase (iNOS) and lowered NO• production by CF respiratory epithelial cells, intrinsically related to the CFTR defect (Kelley & Drumm, 1998). As nitric oxide affects guanylate cyclase activity and cyclic GMP blocks amiloride-sensitive sodium channels including ENaC (Lazrak *et al*, 2000), we wondered whether the sodium-dependent hyperacidification of cellubrevin-positive endosomes in CF can be explained by reduced NO• levels in CF respiratory epithelial cells.

RESULTS

NO• reverses endosomal hyperacidification in CF

Nitric oxide stimulates guanylate cyclase to generate cGMP (Krumenacker *et al*, 2004). As cGMP inhibits amiloride-sensitive sodium channels (Lazrak *et al*, 2000), we tested whether lumenal pH in cellubrevin endosomes could be corrected by supplying NO• to CF cells, as they show lower iNOS levels (Kelley & Drumm, 1998; supplementary Fig S1 online). A pair of previously characterized (Egan *et al*, 1992), genetically matched CF and CFTR-corrected bronchial epithelial cell lines was used: IB3-1 (from a compound heterozygote CF patient with CFTR ΔF508/W1282X alleles) and S9 (IB3-1 cells corrected with a full-size functional CFTR complementary DNA). In addition, CFBE41o− (ΔF508/ΔF508 human bronchial epithelial cells) and 16HBEo− (normal human bronchial epithelial cells) were used. The cells were transfected with a cellubrevin green fluorescent protein (GFP) pHluorin construct to monitor endosomal pH (Miesenbock *et al*, 1998; Poschet *et al*, 2002b). Cellubrevin-containing endosomes of CF cells were hyperacidified compared with endosomes in CFTR-corrected and normal cells (IB3-1, pH 6.1 ± 0.1; S9, pH 6.8 ± 0.1; CFBE41o−, pH 6.1 ± 0.1; 16HBEo−, pH 6.8 ± 0.1; Fig 1A). The NO• donors DETA-NONOate and NOR4 corrected endosomal hyperacidification in IB3-1 cells (1 mM DETA-NONOate, pH 6.9 ± 0.1; 1 mM NOR4, pH 7.0 ± 0.2; Fig 1B), which was further confirmed by examining a dose response to DETA-NONOate (Fig 1C). Conversely to the correction of endosomal pH with NO• donors in CF cells, nitric oxide synthase inhibitors, N^G-nitro-L-arginine methyl ester (L-NAME) and 1400W, induced endosomal hyperacidification in CFTR-corrected S9 cells (1 mM L-NAME, pH 6.5 ± 0.1; 10 μM 1400W, pH 6.0 ± 0.2; Fig 1D). The inactive stereoisomer R-NAME had no effect (1 mM R-NAME, pH 6.8 ± 0.1; Fig 1D). These results indicate that NO• levels affect organellar pH, and that nitric oxide donors can correct endosomal hyperacidification in CF respiratory epithelial cells.

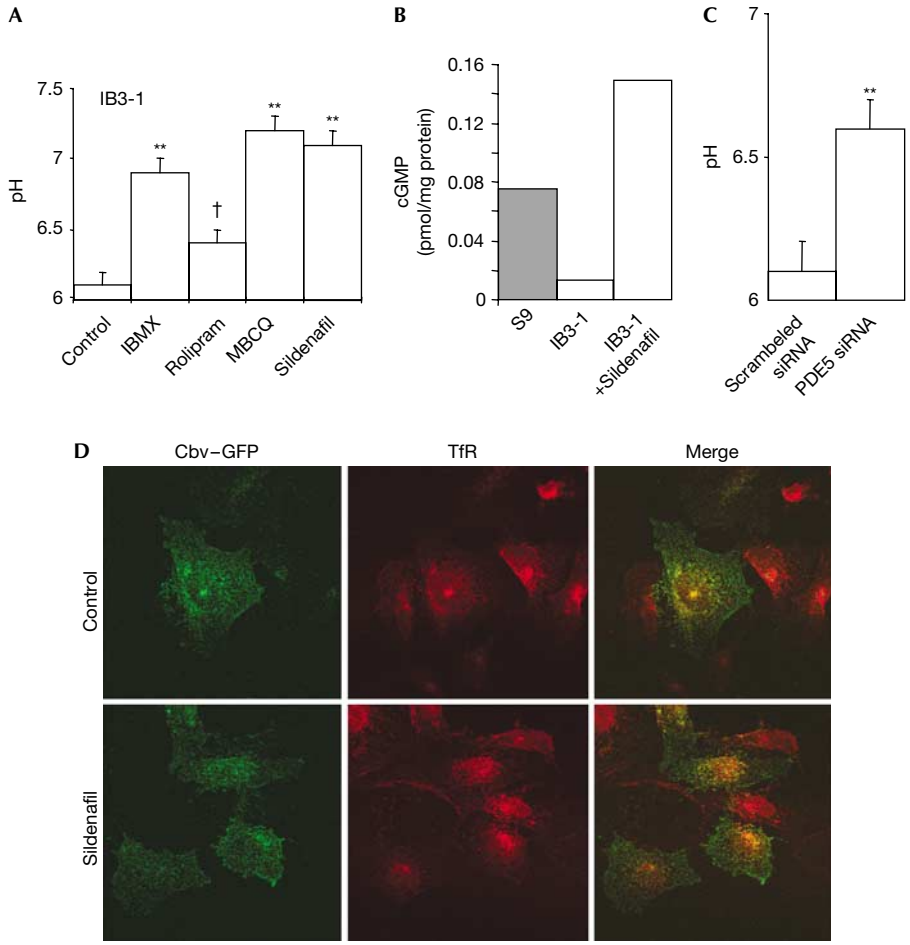

Fig 2 | Inhibition of phosphodiesterase 5 with Sildenafil increases intracellular cGMP and corrects endosomal pH in cystic fibrosis cells. (**A**) IB3-1, pH 6.1 ± 0.1, $n = 18$; IB3-1 + 200 μM IBMX, pH 6.9 ± 0.1, $n = 10$, $P = 0.0002$; IB3-1 + 10 μM Rolipram, pH 6.4 ± 0.1, $n = 10$, $P = 0.1010$; IB3-1 + 100 nM MCBQ, pH 7.2 ± 0.1, $n = 10$, $P = 0.0001$; IB3-1 + 300 nM Sildenafil, pH 7.1 ± 0.1, $n = 10$, $P = 0.0001$. (**B**) IB3-1 cells were treated with 300 nM Sildenafil, lysed and cGMP determined in lyophilized extracts: S9, 0.07 pmol cGMP/mg protein; IB3-1, 0.01 pmol cGMP/mg protein; IB3-1 + 300 nM Sildenafil, 0.15 pmol cGMP/mg protein. (**C**) Endosomal pH in CF cells transfected with scrambled or PDE5 siRNA: IB3-1 + scrambled siRNA, pH 6.1 ± 0.2, $n = 5$; IB3 + PDE5 siRNA, pH 6.6 ± 0.1, $n = 5$, $P = 0.0003$. (**D**) Localization of cellubrevin GFP pHlourin and transferrin receptor in IB3-1 cells ± Sildenafil. TfR shown by immunofluorescence using antibody against human TfR. Note similar overall organellar distribution of cellubrevin GFP probe in both untreated and Sildenafil-treated cells, characterized by partial colocalization with TfR in the perinuclear region where the recycling endosome is located. CF, cystic fibrosis; cGMP, cyclic GMP; PDE5, phosphodiesterase 5; siRNA, short interfering RNA.

Endosomal hyperacidification in CF is reversed by cGMP

As $NO^{\bullet}$ activates guanylate cyclase and cGMP production and cGMP blocks Na^{+} channels (Lazrak *et al*, 2000), we examined potential effects of guanylate cyclase and cGMP on pH of the endosome. The cGMP agonist protoporphyrin IX (PPIX), by increasing cGMP through stimulation of guanylate cyclase, corrected hyperacidification in cellubrevin-containing endosomes of CF cells (IB3-1 + 10 μM PPIX, pH 7.1 ± 0.1; Fig 1E). A similar result was obtained with a soluble guanylate cyclase (sGC) activator BAY41-2272 (IB3-1 + 10 μM BAY41-2272, pH 6.7 ± 0.2). DETA-NONOate did not induce a significant increase in pH when added with the sGC inhibitor NS2028 (Fig 1E). Inhibition of sGC in IB3-1 cells did not further exacerbate the low pH (Fig 1E; bar with ODQ, inhibitor of sGC). Addition of exogenous, membrane-permeant cGMP analogue, 8-Br-cGMP, also reversed endosomal hyperacidification (IB3-1 + 100 μM 8Br-cGMP, pH 7.2 ± 0.1) compared with untreated cells (pH 6.1 ± 0.1; Fig 1F). Addition of another membrane-permeant cGMP derivative, dibutryl cGMP (Freedman & Raff, 1975), reversed hyperacidification (IB3-1 + 100 μM dibutryl cGMP, pH 7.2 ± 0.1; Fig 1F). These results indicate that cGMP corrects hyperacidification of cellubrevin endosomes in CF respiratory epithelial cells.

Phosphodiesterase 5 inhibitors correct endosomal pH

An increase of intracellular cGMP can be achieved by pharmacological inhibition of cGMP hydrolysis to GMP using phosphodiesterase (PDE) inhibitors (Ballard *et al*, 1998). We tested the effects of PDE inhibitors on hyperacidification of cellubrevin endosomes in CF respiratory epithelial cells. Treatment of CF cells with a general PDE inhibitor, IBMX, reversed hyperacidification in

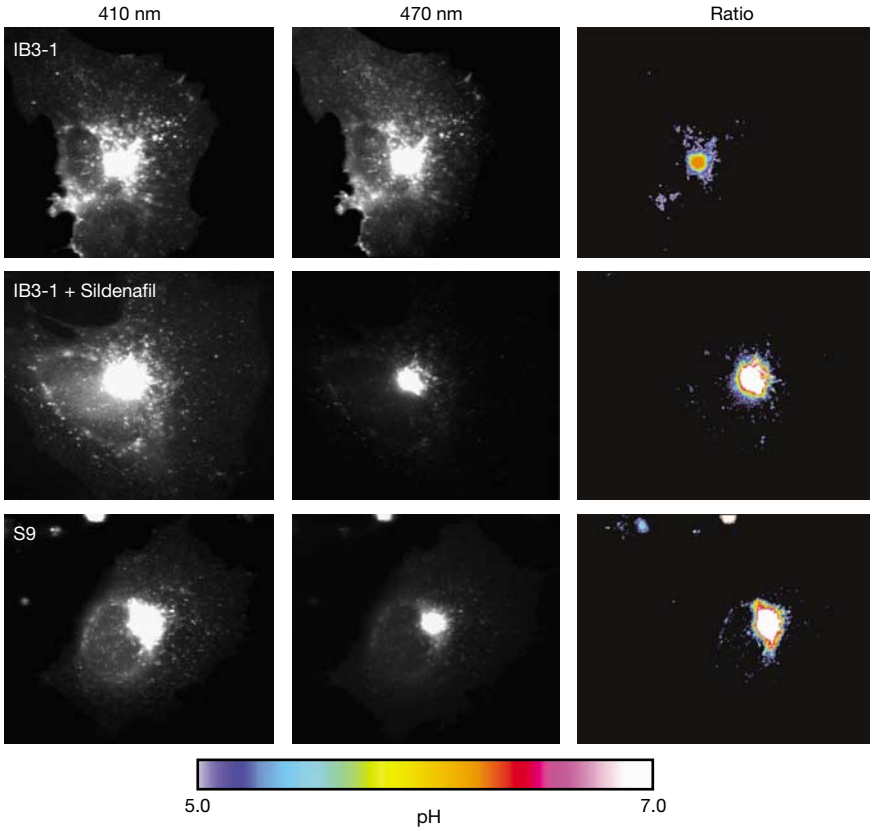

Fig 3 | Live ratiometric fluorescence microscopy of cellubrevin green fluorescent protein pHlourin-labelled endosomes in cystic fibrosis cells treated with Sildenafil. Emission fluorescence was collected/imaged at 508 nm, whereas excitation was rapidly alternated between 410 and 470 nm. Note relative reduction of fluorescence emission after illumination at 470 nm with IB3-1 cells treated with Sildenafil, with intensities closer to those observed with cystic fibrosis transmembrane conductance regulator (CFTR)-corrected cells.

cellubrevin endosomes. IB3-1 treated with 200 μM IBMX had an endosomal pH of 6.9±0.1, relative to a pH of 6.1±0.1 for untreated cells. Inhibition of cyclic AMP (cAMP)-specific PDE with Rolipram did not correct the endosomal hyperacidification (IB3-1 + 10 μM Rolipram, pH 6.4±0.1; Fig 2A), indicating a role for cGMP and not cAMP. Application of a cGMP PDE inhibitor, MBCQ, reversed hyperacidification (IB3-1 + 100 nM MBCQ, pH 7.2±0.1), as did a treatment with the highly specific PDE5 inhibitor Sildenafil (IB3-1 + 300 nM Sildenafil, pH 7.1±0.1; Fig 2A). Treatment with Sildenafil increased intracellular cGMP in IB3-1 cells (Fig 2B). The role of PDE5 in these processes was confirmed by PDE5 knockdown (supplementary Fig S2 online), which increased endosomal pH in CF cells (Fig 2C). Incubation of IB3-1 cells with Sildenafil did not alter localization of cellubrevin GFP pHlourin relative to the transferrin receptor, a marker of recycling endosome (Poschet *et al*, 2002b; Fig 2D). Fig 3 illustrates the fluorescence intensities of pH-sensitive GFP pHlourin after treatment with Sildenafil. We also carried out a converse experiment, whereby Sildenafil reversed a pharmacologically induced endosomal hyperacidification in CFTR-corrected cells S9 (supplementary Fig S3A online).

Finally, we tested primary cells from a lung transplant from a CF patient homozygous for ΔF508 CFTR mutation. Sildenafil treatment reduced the hyperacidification in endosomes of primary human CF respiratory epithelial cells (Fig 4A). Collectively, these results demonstrate that PDE5 inhibitors correct endosomal hyperacidification in CF lung epithelial cells.

Sildenafil reduces IL-8 secretion in CF cells

The proinflammatory neutrophil-recruiting chemokine IL-8 is persistently elevated in CF (Bonfield *et al*, 1995). Endosomes and proinflammatory signalling have previously been linked through a requirement for acidification (Honda *et al*, 2005) in the process of signalling following recognition of endocytosed bacterial products, such as DNA containing unmethylated CpG motifs signalling through Toll-like receptor 9 (TLR9; Latz *et al*, 2004b). It has been shown that CpG DNA induces an inflammatory response in CF cells (Greene *et al*, 2005). We tested whether endosomal hyperacidification in CF cells contributes to the excessive proinflammatory output. Application of Sildenafil to CF cells for 24 h reduced secretion of IL-8 in response to stimulation by DNA preparations from *P. aeruginosa*, a crucial CF pathogen with chromosomal DNA rich in unmethylated CpG motifs (see supplementary Fig S4 online for IL-8 secretion dose–response curve as a function of increasing concentrations of *P. aeruginosa* DNA). Mean IL-8 secretion for IB3-1 cells was 161±26 pg/ml and for IB3-1 + 300 nM Sildenafil was 89±23 pg/ml, $P = 0.0221$ (Fig 4B). Sildenafil was also able to suppress IL-8

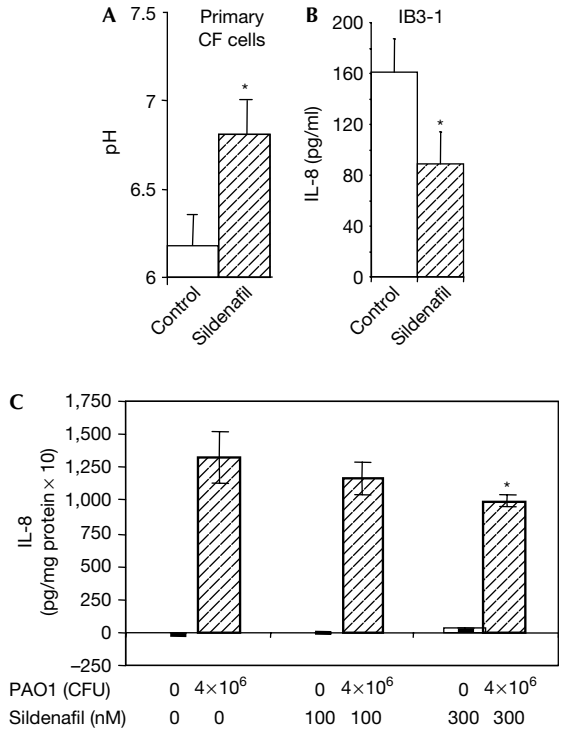

Fig 4 | Sidlenafil normalizes endosomal pH in primary cystic fibrosis human respiratory epithelial cells and reduces interleukin 8 secretion in cystic fibrosis cells in response to stimulation with *Pseudomonas aeruginosa* products. (**A**) Correction of endosomal pH by Sildenafil in human primary respiratory epithelial cells from a cystic fibrosis lung transplant (ΔF508/ΔF508 homozygous patient); $n = 5$, $P = 0.0173$. (**B**) IB3-1 cells were treated with 25 μg/ml *P. aeruginosa* DNA as a proinflammatory stimulant. Interleukin 8 (IL-8) secreted by cells was determined by enzyme-linked immunosorbent assay. (**C**) IB3-1 cells were incubated for 3 h with media alone (open bar), or exposed to 4×10^6 CFU/well of live *P. aeruginosa* PAO1 (striped bars). In Sildenafil-treated samples, cells were preincubated in its presence or absence for 3 h before stimulation. After infection, cells were washed and incubated with media in the presence of 100 μg/ml of gentamicin for 21 h. IL-8 was measured in supernatants by ELISA. Values represent means and standard errors. *$P < 0.05$ (analysis of variance).

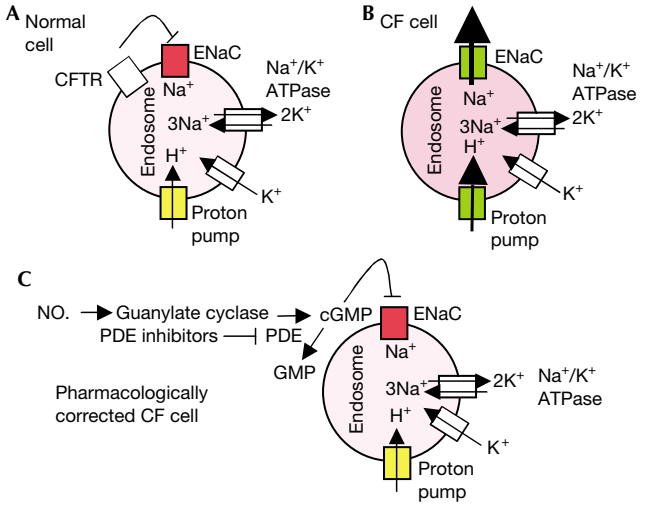

Fig 5 | Model of endosomal hyperacidification in cystic fibrosis respiratory epithelial cells and its correction by NO• and phosphodiesterase 5 inhibitors. Shades of pink, endosome lumenal acidification levels (darker colour—lower pH); green boxes, active pumps or open channels; red boxes, inactive channels; yellow boxes, normal pump or channel activity. (**A**) In normal human respiratory epithelial cells, the CFTR inhibits sodium channels (ENaC), resulting in positive charge build-up as vacuolar H^+ ATPase pumps protons into the endosomal lumen. The proton pump is sensitive to transmembrane potential build-up, and shuts down, resulting in physiologically normal, mild lumenal acidification of the endosome. (**B**) In CF respiratory epithelial cells, with defective CFTR, ENaC is no longer inhibited by CFTR, which allows efflux of sodium as the protons are being pumped into the endosomal lumen by the proton pump. The sodium efflux dissipates membrane potential, allowing the vacuolar H^+ ATPase to extend its proton-pumping action, thus causing mild hyperacidification. (**C**) As shown in this work, nitric oxide (NO•) can correct endosomal hyperacidification in CF respiratory epithelial cells, by cGMP-dependent block of sodium transport through amiloride-sensitive channel ENaC. Hyperacidification can be corrected using Sildenafil (Viagra) or other inhibitors specific for cGMP PDE5. For further information, see Note S1 of the supplementary information online. CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; cGMP, cyclic GMP; PDE5, phosphodiesterase 5.

production in IB3-1 cells following stimulation with live *P. aeruginosa* (Fig 4C). Sildenafil alone did not stimulate secretion of IL-8 in cells that were not exposed to *P. aeruginosa* products (supplementary Fig S5 online). Thus, Sildenafil reduces secretion of IL-8 in CF respiratory epithelial cells when they are exposed to whole *P. aeruginosa* bacteria or are stimulated with *P. aeruginosa* DNA, consistent with Sildenafil effects on endosomal pH.

DISCUSSION

Hyperacidification of endosomal compartments in CF respiratory epithelial cells is due to dysregulated sodium transport in CFTR mutant cells (Poschet *et al*, 2002a). Here, we have shown that drugs promoting the formation or preventing the degradation of cGMP reverse endosomal hyperacidification in CF cells. This includes NO• donors, which stimulate guanylate cyclase to produce more cGMP (Roczniak & Burns, 1996), or PDE inhibitors, which prevent the degradation of cGMP (Ballard *et al*, 1998). Fig 5 shows a model summarizing these relationships.

Before the more recent demonstration of organellar hyperacidification in CF respiratory epithelial cells (Chandy *et al*, 2001; Poschet *et al*, 2001, 2002a,b), there were proposals that the absence of the CFTR might lead to alkalinization of the intracellular organellar lumen owing to a loss of Cl^- conductance (Barasch *et al*, 1991). Later research found no organellar alkalinization (Biwersi & Verkman, 1994; Seksek *et al*, 1996; see the supplementary information online for detailed discussion). Subsequent studies have confirmed that organelles in CF respiratory epithelial cells are not more alkaline, and instead have demonstrated that the TGN and recycling endosomes are hyperacidified in human CF respiratory epithelial cells

(Chandy *et al*, 2001; Poschet *et al*, 2001, 2002a,b). These studies have shown that organellar hyperacidification in CF respiratory cells is due to the loss of regulation of sodium transport in the absence of a functional CFTR, and the runaway flux of Na^+ out of the organelles (Poschet *et al*, 2002a). Further support for this model (Fig 5) is provided (supplementary information online) by inhibition of Na^+/K^+ ATPase (supplementary Fig S3A online) and amiloride-sensitive Na^+ channels (supplementary Fig S3B online).

The expression of iNOS is reduced in CF airway epithelial cells (Kelley & Drumm, 1998) and in human lung explants, but not in other lung diseases (Meng *et al*, 1998). In their initial publication on decreased iNOS levels, Kelley & Drumm (1998) have proposed the possibility that decreased $NO^\bullet$ levels in CF may be linked to aberrant Na^+ transport in the CF respiratory epithelium through the decreased action of $NO^\bullet$ on guanylate cyclase. Our work presented here indirectly validates their model, as reflected in organellar hyperacidification in CF cells. This signalling cascade is the basis for Sildenafil action in our experiments, and suggests potential therapeutic uses, in keeping with reports linking Sildenafil action on the $NO^\bullet$/cGMP pathway to positive outcomes in animal models of lung disease (Toward *et al*, 2004; Ladha *et al*, 2005).

CF is characterized by excessive lung inflammation. Treatment of CF cells with Sildenafil reduced secretion of the proinflammatory chemokine IL-8 elicited by stimulation with high CpG DNA of *P. aeruginosa*. Endosomal pH is important in proinflammatory TLR9 signalling, as treatment with bafilomycin or chloroquine abrogates this signalling (Honda *et al*, 2005). In macrophages and dendritic cells, CpG DNA is internalized by endocytosis, whereas TLR9 moves from the endoplasmic reticulum to endosomal organelles where it binds to CpG DNA and activates signalling (Latz *et al*, 2004a). Hyperacidification of endosomes in CF respiratory epithelial cells augments proinflammatory signalling in response to *Pseudomonas* products, as shown previously (Firoved *et al*, 2004) and further extended here. Sildenafil treatment, as predicted from the normalization of endosomal pH, resulted in normalization of IL-8 secretion. Thus, not only does this work show a pathway leading to the hyperacidification of proinflammatory signalling organelles in CF, but also offers a remedy using a safe and widely used drug.

METHODS

Live fluorescence ratiometric microscopy and pH measurements. Fluorescence microscopy was as previously described (Poschet *et al*, 2002b) and was performed by using an Olympus IX-70 microscope and Olympix KAF1400 CCD camera (LSR, Olympus, NY, USA). Following excitation at 410 and 470 nm, the ratio of emission at 508 nm was determined using filter sets (Chroma, VT, USA) mounted in a Sutter filter wheel (Sutter Instruments, CA, USA) and processed by Ultraview software (Perkin-Elmer, CA, USA). Cells were mounted in a perfusion chamber in buffer A: 25 mM HEPES (pH 7.4), 119 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 30 mM glucose at 37 °C. External and internal standards and calibration curve were generated as previously described (Poschet *et al*, 2001, 2002b).

Modulation of cellubrevin endosomal pH. After 72 h from the time of transfection, cells were treated with drugs for 20 min to 2 h at 37 °C and 5% CO_2, and cellubrevin endosomal pH was determined as described above.

Other methods. Further methods are described in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

This work was supported by a Cystic Fibrosis Foundation grant (to G.S.T.), National Institutes of Health grants AI 50825 and AI 31139, and in part by Philip Morris USA, Inc., and by Philip Morris International (to V.D.). W.O. was a Cystic Fibrosis Foundation Fellow.

REFERENCES

Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, Naylor AM (1998) Effects of sildenafil on the relaxation of human corpus cavernosum tissue *in vitro* and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol* **159:** 2164–2171

Barasch J, Kiss B, Prince A, Saiman L, Gruenert D, al-Awqati Q (1991) Defective acidification of intracellular organelles in cystic fibrosis. *Nature* **352:** 70–73

Biwersi J, Verkman AS (1994) Functional CFTR in endosomal compartment of CFTR-expressing fibroblasts and T84 cells. *Am J Physiol* **266:** C149–C156

Bonfield TL, Panuska JR, Konstan MW, Hilliard KA, Hilliard JB, Ghnaim H, Berger M (1995) Inflammatory cytokines in cystic fibrosis lungs. *Am J Respir Crit Care Med* **152:** 2111–2118

Chandy G, Grabe M, Moore HP, Machen TE (2001) Proton leak and CFTR in regulation of Golgi pH in respiratory epithelial cells. *Am J Physiol Cell Physiol* **281:** C908–C921

Cutting GR (2005) Modifier genetics: cystic fibrosis. *Annu Rev Genomics Hum Genet* **6:** 237–260

Egan M, Flotte T, Afione S, Solow R, Zeitlin PL, Carter BJ, Guggino WB (1992) Defective regulation of outwardly rectifying Cl^- channels by protein kinase A corrected by insertion of CFTR. *Nature* **358:** 581–584

Firoved AM, Ornatowski W, Deretic V (2004) Microarray analysis reveals induction of lipoprotein genes in mucoid *Pseudomonas aeruginosa*: implications for inflammation in cystic fibrosis. *Infect Immun* **72:** 5012–5018

Freedman MH, Raff MC (1975) Induction of increased calcium uptake in mouse T lymphocytes by concanavalin A and its modulation by cyclic nucleotides. *Nature* **255:** 378–382

Gentzsch M, Chang XB, Cui L, Wu Y, Ozols VV, Choudhury A, Pagano RE, Riordan JR (2004) Endocytic trafficking routes of wild type and ΔF508 cystic fibrosis transmembrane conductance regulator. *Mol Biol Cell* **15:** 2684–2696

Greene CM, Carroll TP, Smith SG, Taggart CC, Devaney J, Griffin S, O'Neill SJ, McElvaney NG (2005) TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. *J Immunol* **174:** 1638–1646

Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, Taya C, Taniguchi T (2005) Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature* **434:** 1035–1040

Kelley TJ, Drumm ML (1998) Inducible nitric oxide synthase expression is reduced in cystic fibrosis murine and human airway epithelial cells. *J Clin Invest* **102:** 1200–1207

Krumenacker JS, Hanafy KA, Murad F (2004) Regulation of nitric oxide and soluble guanylyl cyclase. *Brain Res Bull* **62:** 505–515

Ladha F, Bonnet S, Eaton F, Hashimoto K, Korbutt G, Thebaud B (2005) Sildenafil improves alveolar growth and pulmonary hypertension in hyperoxia-induced lung injury. *Am J Respir Crit Care Med* **172:** 750–756

Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, Lien E, Nilsen NJ, Espevik T, Golenbock DT (2004a) TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* **5:** 190–198

Latz E, Visintin A, Espevik T, Golenbock DT (2004b) Mechanisms of TLR9 activation. *J Endotoxin Res* **10:** 406–412

Lazrak A, Samanta A, Matalon S (2000) Biophysical properties and molecular characterization of amiloride-sensitive sodium channels in A549 cells. *Am J Physiol Lung Cell Mol Physiol* **278:** L848–L857

Meng QH *et al* (1998) Lack of inducible nitric oxide synthase in bronchial epithelium: a possible mechanism of susceptibility to infection in cystic fibrosis. *J Pathol* **184:** 323–331

Miesenbock G, De Angelis DA, Rothman JE (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* **394:** 192–195

Poschet JF, Boucher JC, Tatterson L, Skidmore J, Van Dyke RW, Deretic V (2001) Molecular basis for defective glycosylation and *Pseudomonas* pathogenesis in cystic fibrosis lung. *Proc Natl Acad Sci USA* **98:** 13972–13977

Poschet JF, Perkett E, Deretic V (2002a) Hyperacidification in cystic fibrosis: links with lung disease and new prospects for treatment. *Trends Mol Med* **8:** 512–519

Poschet JF, Skidmore J, Boucher JC, Firoved AM, Van Dyke RW, Deretic V (2002b) Hyperacidification of cellubrevin endocytic compartments and defective endosomal recycling in cystic fibrosis respiratory epithelial cells. *J Biol Chem* **277:** 13959–13965

Riordan JR (2005) Assembly of functional CFTR chloride channels. *Annu Rev Physiol* **67:** 701–718

Roczniak A, Burns KD (1996) Nitric oxide stimulates guanylate cyclase and regulates sodium transport in rabbit proximal tubule. *Am J Physiol* **270:** F106–F115

Rowe SM, Miller S, Sorscher EJ (2005) Cystic fibrosis. *N Engl J Med* **352:** 1992–2001

Seksek O, Biwersi J, Verkman AS (1996) Evidence against defective *trans*-Golgi acidification in cystic fibrosis. *J Biol Chem* **271:** 15542–15548

Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, Boucher RC (1995) CFTR as a cAMP-dependent regulator of sodium channels. *Science* **269:** 847–850

Toward TJ, Smith N, Broadley KJ (2004) Effect of phosphodiesterase-5 inhibitor, sildenafil (Viagra), in animal models of airways disease. *Am J Respir Crit Care Med* **169:** 227–234